REMARKS

Claims 1-53 are pending. Applicants have amended claim 1 to exclude "hydroxyl" from the listing of permissible substituents for variables R_1, R_2, R_3, R_4, R_5, R_6, R_7, R_8, R_9, R_{10}, R_{11}, R_{12}, and R_{13}. Applicants have also amend claims 46-48 and 51-53 to comport in scope with claim 1 as presently amended. Support for this amendment can be found throughout the specification (*e.g.*, *see* Examples 37-52 and 54-59). No new matter has been added.

35 U.S.C. § 112 ¶2

Claims 1-53 are rejected as allegedly being indefinite for including the term "DNA minor groove binder." The Examiner recognizes that the specification "has some formulas and some examples," but argues that this does not clearly define the metes and bounds of the claims (Office action at page 2). The Examiner further states that the term in question encompasses a "myriad of compounds and ... represents only an invitation to experiment regarding possible compounds" (Office action at page 2).

Applicants respectfully traverse this ground for rejection. First, the MPEP makes it very clear that breadth alone is not equivalent to indefiniteness. The Examiner's attention is kindly directed to 2173.04, which states, *[b]readth of a claim is not to be equated with indefiniteness.* (emphasis added). Thus, whether or not the claim encompasses a "myriad of compounds" is not relevant. Second, Applicants know of no reason why "an invitation to experiment" should be considered under this statutory provision; such considerations are not part of the analysis for clarity. What should be considered is whether the claims "set out and circumscribe" the claimed compounds "with *a reasonable degree* of clarity and particularity." MPEP at 2173 (emphasis added). "The primary purpose of this requirement of definiteness of claim language is to ensure that the scope of the claims is clear so the public is informed of the boundaries of what constitutes infringement of the patent." MPEP at 2173. The standard for concluding that claim language is indefinite is quite high. *Metabolite Labs., Inc. v. Lab Corp. of America Holdings*, 370 F.3d 1354 (Fed. Cir. 2004) ("Only when a claim remains insolubly ambiguous without a discernible meaning after all reasonable attempts at construction must a court declare it indefinite.")

The Examiner should consider (a) the specification; (b) the teachings of the prior art; and (c) the interpretation one of ordinary skill in the art would reach at the time the invention was made. MPEP at 2173.

Here, the specification teaches that "the DNA minor groove binder can have the formula, $-CONH(CH_2)_r$-J-W-$(CH_2)_tR^e$, in which ..." (*see* page 10, line 25, through page 12, line 3). Examples of chemical structures for DNA minor groove binders such as formulas (III) and (IV) are also provided. Moreover, the term "DNA minor groove binder" is known in the art. It refers to a class of anticancer agents useful in treating a variety of human cancers. Fedlier *et al.* (*Br. J. Cancer* 89:1559-1565, 2003 (enclosed) begins their introduction as follows:

> Minor groove binders (MGBs) represent an interesting class of anticancer agents, which have been shown to be highly effective in *in vitro* and *in vivo* preclinical tumour models unresponsive to other antineoplastic agents ...

In view of the present specification and the teaching of the prior art at the time the present application was filed, one of ordinary skill in the art would be able to determine, with *a reasonable degree* of certainty whether or not a compound they wished to make and use would fall within the scope of the present claims. Accordingly, this ground for rejection should be withdrawn.

35 U.S.C. § 112 ¶ 1

Claims 1-53 are rejected for lack of enablement. The Examiner acknowledges that the specification is enabling for some compounds with $CONHC(CH_2)_2NMe_2$, but alleges that the specification "does not reasonably provides enablement for 1) any and all the substituents such as DNA minor grove binder 2) for all the R's to be substituted with these large groups 3) for the compounds to be able to treat any and all cancers."

The Examiner goes on to comment on several of the factors set out in *in re Wands*, 858 F.2d 731 (Fed. Cir. 1988). For example, regarding the breadth of the claims, the Examiner states, "[t]he instant claims encompass many compounds *with a different core* and different groups hanging off of it" (Office action at page 3; emphasis added). This is characterization is not understood. While the claims do cover many compounds, they must all

conform to the four-ring backbone of formula (I), and the specification provides substantial guidance as to how such compounds can be synthesized. For example, Scheme 6 at page 20 illustrates the preparation of compounds with DNA minor groove binders, and Applicants' teaching at pages 18 and 19 provides detailed synthesis procedures for the DNA minor groove binders. More specifically, Scheme 6 shows that the introduction of the DNA minor groove binders can be achieved by coupling acyl chloride 32 and amine 33. A person of ordinary skill in the art would then know how to prepare a compound with such substituents. Furthermore, the present specification describes how to make the final compound of Example 59, which contains a DNA minor groove binders. The specification also teaches how to synthesize the DNA minor groove binder moiety as shown in Examples 39 and 40. Based on the information in the specification, a person of ordinary skill in the art would know how to prepare a compound with such substituents.

Regarding predictability, the Examiner argues that "the structure of the compound has to be specific" and that "[e]ven a difference of a methyl group verses a hydrogen" can change a compound's properties altogether (Office action at page 3). In response, the Examiner is asked to note that: (a) absolute predictability is not required for enablement and (b) one of ordinary skill in the art is well able to determine whether and how changes to various substituents exert their effect. The standard for enablement is not whether experimentation is required, but rather, whether in the context of the relevant art, that experimentation would be undue. *In re Wright*, 999 F.2d 1557, 1561 (Fed. Cir. 1993). Further, and as the Examiner recognizes, the level of skill in the art is high.

Regarding the use of the compounds of the present invention to treat cancers, the Examiner states that "[t]here is no absolute predictability and no established **correlation** between in vitro activity and the treatment of diseases as the in vitro data is not a reliable predictor of success ....." The Examiner further states that "[t]he greatly increased complexity of the in vivo environment as compared to the very narrowly defined and controlled conditions of an in-vitro assays does not permit a single extrapolation of in vitro assays to human diagnostic efficacy with any reasonable degree of predictability." See the Office Action at page 4.

According to the MPEP at 2164.02,

> the issue of "correlation" is also dependent on the state of the prior art. In other words, if the art is such that a particular model is recognized as correlating to a specific condition, then it should be accepted as correlating unless the examiner has evidence that the model does not correlate. Even with such evidence, the examiner must weigh the evidence for and against correlation and decide whether one skilled in the art would accept the model as reasonably correlating to the condition. *In re Brana*, 51 F.3d 1560, 1566, 34 USPQ2d 1436, 1441 (Fed. Cir. 1995)(reversing the PTO decision based on finding that *in vitro* data did not support *in vivo* applications).
>
> [B]ased upon the relevant evidence as a whole, there is a reasonable correlation between the disclosed *in vitro* utility and an *in vivo* activity, and therefore a rigorous correlation is not necessary where the disclosure of pharmacological activity is reasonable based upon the probative evidence. (Citations omitted.)

The present specification provides art recognized *in vitro* tumor cell growth inhibition assays to evaluate the antitumor activity of the claimed compounds. All final compounds (Examples 37-58) except Example 59 were tested for antitumor activity and were shown to have activity. Compound 37 was also tested in nude mice bearing human breast tumor, MX-1, xenografts and bearing human T-cell leukemic lymphoma CCRF-CEM xenografts and resulted in the reduction of tumor size. Accordingly, one skilled in the art at the time of filing would have reasonably predicted that the claimed compounds would have been useful for treating cancer including.

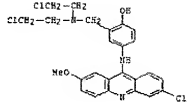
In view of the foregoing, Applicants respectfully request the Examiner reconsider and withdraw the rejection under 35 U.S.C. § 112 ¶1.

35 U.S.C. § 102(b)

Claims 1-3, 6-11, 13, 16-19, 22-26, 29, 30, 39-43, 46-49, and 51 are rejected as allegedly being anticipated by Elslager *et al.* (U.S. Patent No. 2,882,382; "Elslager").

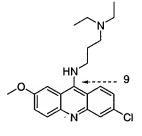
In view of the present amendment, the Examiner is asked to reconsider and withdraw this ground for rejection. Applicants have amended claim 1 to exclude "hydroxyl" from the list of permissible substituents for the variables R_1, R_2, R_3, R_4, R_5, R_6, R_7, R_8, R_9, R_{10}, R_{11}, R_{12}, and R_{13}. Thus, none of these variables can be a hydroxyl group.

Elslager discloses the following compound:



As can be seen, the substituent corresponding to Applicant's R_3 in the Elslager compound is a hydroxyl group. As noted, in claim 1 as presently amended, R_3 cannot be a hydroxyl group. Therefore, Elslager's compound is not within the scope of claim 1 as presently amended and, thus, cannot anticipate the compounds of claim 1. As the claims that depend from claim 1 incorporate the limitations of claim 1, they are similarly free of Elslager. Accordingly, this ground for rejection should now be withdrawn.

Claims 1-3, 6-11, 13, 16-19, 22-26, 29, 30, 39-43, 46-49, and 51-53 are rejected as being anticipated by Joseph Leiter, 1960, Cancer Chemotherapy Screening Data (hereafter "Leiter"). More specifically, the Examiner states that Leiter discloses compound 353 at page 554. It appears that the Examiner has provided an incorrect structure of compound 353, because the structure provided in the Office action (at page 8) is identical to the structure relied on in Elslager. Applicants' representatives used ChemDraw Ultra 9.0 to generate the Leiter compound as:



3-chloro-9-(3-di-ethylaminopropylamino)-7-methoxy-acridine

This compound is structurally different from the compounds of claim 1. The compounds of the present claims require an arylamine moiety at the 9-position (shown above), while the Leiter compound contains a di-ethylaminopropylamino group at that position. Accordingly, the

Leiter compound falls outside the scope of claim 1 and cannot anticipate claim 1 or any claim that depends therefrom.

35 U.S.C. § 103(a)

Claims 1-31 and 39-53 are rejected as being obvious over Leiter. The Examiner again refers to compound 353 of Leiter but reproduces the structure shown in the other reference -- Elslager (see the Office action at page 10). After stating that "[t]he substituents are the same as those of the invention" the Examiner goes on, under the heading "*Difference between Prior Art and the claims*" to state:

> The applicants invention claims these substitutions can be anywhere for R1-R13. Making them positional isomers and positional isomers are considered prima facie obvious in the absence of unexpected results. According to KSR v Teleflex one of skill in the art would have found it obvious and be motivated to make positional isomers with a reasonable expectation of success that it would retain its pharmaceutical properties.

This ground for rejection is respectfully traversed. The compounds Applicants now claim conform to Formula (I), with the proviso that R3 cannot be hydroxyl. Neither Elslager nor Leiter disclose or suggest such a compound, nor are the claimed compounds positional isomers of the Elslager compound or compound 353 of Leiter. As noted above, the Leiter compound is substantially different from the compounds claimed; the Leiter compound having a $NHCH_2CH_2CH_2NMe_2$ group at the 9-positon, where the compounds claimed have a ring structure -- an arylamine moiety. Thus, the prior art compounds are not simply positional isomers of the presently claimed compounds, and there is no reason why one of ordinary skill in the art would have been motivated to modify compound 353 to include the fourth ring required by the present claims. In view of the foregoing, it is Applicants position that the rejection for obviousness must be withdrawn.

CONCLUDING FORMALITIES

No fee is believed due in connection with this amendment. If there are any charges, or any credits, please apply them to deposit account 06-1050, referencing Attorney Docket No. 08919-0118001.

Respectfully submitted,

Date: September 19, 2008

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Brostallicin (PNU-166196) – a new DNA minor groove binder that retains sensitivity in DNA mismatch repair-deficient tumour cells

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Defects in DNA mismatch repair (MMR) are associated with a predisposition to tumorigenesis and with drug resistance owing to high mutation rates and failure to engage DNA-damage-induced apoptosis. DNA minor groove binders (MGBs) are a class of anticancer agents highly effective in a variety of human cancers. Owing to their mode of action, DNA MGB-induced DNA damage may be a substrate for DNA MMR. This study was aimed at investigating the effect of loss of MMR on the sensitivity to brostallicin (PNU-166196), a novel synthetic α-bromoacrylic, second-generation DNA MGB currently in Phase II clinical trials and structurally related to distamycin A. Brostallicin activity was compared to a benzoyl mustard derivative of distamycin A (tallimustine). We report that the sensitivities of MLH1-deficient and -proficient HCT116 human colon carcinoma cells were comparable after treatment with brostallicin, while tallimustine resulted in a three times lower cytotoxicity in MLH1-deficient than in -proficient cells. MSH2-deficient HEC59 parental endometrial adenocarcinoma cells were as sensitive as the proficient HEC59 + ch2 cells after brostallicin treatment, but were 1.8-fold resistant after tallimustine treatment as compared to the MSH2-proficient HEC59 + ch2 counterpart. In addition, p53-deficient mouse fibroblasts lacking PMS2 were as sensitive to brostallicin as PMS2-proficient cells, but were 1.6-fold resistant to tallimustine. Loss of neither ATM nor DNA-PK affected sensitivity to brostallicin in p53-deficient mouse embryonic fibroblasts, indicating that brostallicin-induced cytotoxicity in a p53-deficient genetic background does not seem to require these kinases. These data show that, unlike other DNA MGBs, MMR-deficient cells retain their sensitivity to this new α-bromoacrylic derivative, indicating that brostallicin-induced cytotoxicity does not depend on functional DNA MMR. Since DNA MMR deficiency is common in numerous types of tumours, brostallicin potentially offers the advantage of being effective against MMR-defective tumours that are refractory to several anticancer agents.

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Keywords: brostallicin; DNA mismatch repair; drug sensitivity; DNA minor groove binder

Minor groove binders (MGBs) represent an interesting class of anticancer agents, which have been shown to be highly effective in *in vitro* and *in vivo* preclinical tumour models unresponsive to other antineoplastic agents (Martin *et al*, 1981; Li *et al*, 1982, 1992; Hartley *et al*, 1988; D'Alessio *et al*, 1994; D'Incalci, 1994; Colella *et al*, 1999; Marchini *et al*, 1999; Geroni *et al*, 2002). The main representatives of this class, which reached the clinic, are the antitumour agents derived from CC-1065, that is, adozelesin, carzelesin, and bizelesin, and the distamycin A derivative tallimustine. These 'classical' MGBs have been shown to be highly DNA sequence-specific (Lee *et al*, 1993; D'Incalci, 1994) and to exert their cytotoxic effect through the ability to *per se* directly alkylate DNA mainly at the N3 position of adenines exposed in (TA)-rich sequences in the DNA minor groove (Hurley *et al*, 1984; Reynolds *et al*, 1985; Broggini *et al*, 1991, 1995; Sun and Hurley, 1992; D'Incalci, 1994; Marchini *et al*, 1998), without the requirement to be activated by other pathways (e.g., enzymatic activation of the drug). The absence of significant antitumour activity for nonalkylating MGBs (Marchini *et al*, 1998) indicates that the N3 alkylation activity of these compounds is a prerequisite for their cytotoxicity. MGBs activity, however, has previously been reported (Colella *et al*, 1999) to be associated with reduced susceptibility to the cytotoxic effect in tumour cells with defects in DNA mismatch repair (MMR), similar to certain chemicals, including MNNG, which alkylates O6 of guanines, and anticancer agents such as doxorubicin and cisplatin (Branch *et al*, 1995; Drummond *et al*, 1996).

MMR proteins recognise mismatched base pairs in the DNA, arising either spontaneously during DNA metabolism or from modified nucleotides provoked by physical and chemical agents, and are thought to link DNA damage recognition to an apoptotic pathway, thereby preventing mutagenesis, tumorigenesis, and tumour progression (Modrich, 1991; Fink *et al*, 1998). Tumours resulting from MMR-deficiency include the hereditary nonpolyposis colon cancer (HNPCC) and some sporadic carcinomas such as mammary, ovarian, or endometrial cancers (Peltomaki, 2001). The development of novel MGBs able to overcome the involvement of MMR assumes great clinical importance with respect to the treatment of tumours deficient in MMR. A novel α-bromoacryloyl derivative of distamycin A, PNU-151807, which exhibits no

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alkylating activity *per se*, has been identified (Marchini *et al*, 1999). The cytotoxic effect has been shown to not depend on MLH1 in some tumour cells (Colella *et al*, 1999) and has been attributed to the α-bromoacrylic moiety of the compound, which seems to interfere with cell cycle progression via yet unknown pathways (Cozzi, 2000; Geroni *et al*, 2002).

Recently, brostallicin (PNU-166196), a synthetic α-bromoacrylic MGB structurally related to PNU-151807, has been selected for clinical development. Brostallicin has shown very promising activity in experimental tumour models; its *in vitro* and *in vivo* activity is increased in tumour cells with higher glutathione (GSH) and/or glutathione-S-transferase (GST) levels (Geroni *et al*, 2002). The α-bromoacrylic moiety of brostallicin was found to react with GSH, in a reaction catalysed by GST, with the possible formation of a highly reactive GSH-complex able to bind covalently to DNA (Geroni *et al*, 2002; Cozzi, 2003).

The present study was aimed at investigating the effect of loss of MMR on the sensitivity to brostallicin compared to the structurally related tallimustine, using cell lines deficient or proficient in MLH1, MSH2, or PMS2, respectively. A putative involvement of two members of the PI3-like kinase family, ATM and DNA-PK, which link DNA damage and cell cycle response in drug-induced cytotoxicity, was also investigated. We report that MMR-deficient cells retain sensitivity to brostallicin, thereby extending the list of potential anticancer agents for use in the treatment of MMR-deficient tumours, and that brostallicin-induced cytotoxicity may not require ATM and DNA-PK.

MATERIALS AND METHODS

Cell lines

The MLH1-deficient human colorectal adenocarcinoma cell line HCT116 was obtained from the American Type Culture Collection (ATCC CCL 247), and a subline complemented with chromosome 3 carrying the wild-type gene for *hMLH1* (clone HCT116/3–6, identified here as HCT116 + ch3) was obtained from Dr M Koi (Koi *et al*, 1994) as were the MSH2-deficient human endometrial adenocarcinoma cell line HEC59 (Umar *et al*, 1997) and a subline complemented with chromosome 2 carrying the wild-type gene for *hMSH2* (clone HEC59/2–4, identified here as HEC59 + ch2). HCT116 cells contain a hemizygous mutation in *MLH1* resulting in a truncated, nonfunctional protein (Boyer *et al*, 1995). Similarly, the HEC59 cells are mutated at different loci on both alleles of *MSH2* and are deficient in repair activity (Umar *et al*, 1997). The chromosome-complemented sublines HCT116 + ch3 and HEC59 + ch2 are competent in DNA MMR. HCT116 and HEC59 cell lines were maintained in Iscove's modified Dulbecco's medium (Life Technologies, Basel, Switzerland) supplemented with 2 mM L-glutamine and 10% heat-inactivated foetal bovine serum (Oxoid, Basel, Switzerland). The chromosome-complemented lines were maintained in a medium supplemented with geneticin (400 $\mu g\,ml^{-1}$ for HCT116 + ch3, and 600 $\mu g\,ml^{-1}$ for HEC59 + ch2) (Life Technologies). Although the extent of possible effects of the introduction of an extra chromosome are not fully clear, it is generally acknowledged that it does not spoil the effects of loss of MMR on drug sensitivity. PMS2$^{-/-}$/p53$^{-/-}$ and PMS2$^{+/+}$/p53$^{-/-}$ cell lines, established from E1A/Ha-Ras-transformed knockout mice primary fibroblasts, were generously provided by Dr P Glazer (Zeng *et al*, 2000). Cells are maintained in culture for a limited number of passages and are routinely tested for the expression of MMR proteins. The ATM$^{+/+}$/p53$^{-/-}$ and ATM$^{-/-}$/p53$^{-/-}$, and the DNA-PK$^{+/+}$/p53$^{-/-}$ and DNA-PK$^{-/-}$/p53$^{-/-}$ mouse embryonic fibroblasts, were generously provided by Dr P Leder (Westphal *et al*, 1997) and Dr EH Goodwin (Bailey *et al*, 1999), respectively. The cells were maintained in DMEM medium supplemented with 2 mM L-glutamine (Life Technologies), 10% heat-inactivated foetal calf serum (Oxoid) and penicillin/streptomycin (100 U ml^{-1}/100 $\mu g\,ml^{-1}$, Life Technologies). All cell lines were tested negative for contamination with *Mycoplasma* spp. and maintained in a controlled environment of 5% CO_2 and 95% relative humidity at 37°C. Except for the ATM$^{+/+}$/p53$^{-/-}$ and ATM$^{-/-}$/p53$^{-/-}$ mouse cells, which grow as a monolayer and do not form colonies, all other cell lines used in these experiments form well-defined individual colonies when seeded sparsely on standard tissue culture plates.

Reagents

Distamycin A and its derivatives brostallicin (PNU-166196) and tallimustine (PNU-152241) were synthesised by Pharmacia Italy (Nerviano, Italy). The chemical structures of the derivatives are presented in Figure 1. Brostallicin was dissolved in methanol, tallimustine in DMSO, and distamycin A in water. Stock solutions were stored at −20°C. The final concentration of DMSO or methanol in the cultures was <0.1% at all drug concentrations and in controls. Previous experiments (data not shown) have shown that neither 0.1% DMSO nor 0.1% methanol affects the viability or growth of these cell lines.

MPE footprinting analysis

The MPE footprinting method has been previously described in detail (Hertzberg and Dervan, 1984). The 4492- and 751-bp fragments of SV40-labelled plasmid previously described (Marchini *et al*, 1999) were incubated with distamycin A, tallimustine, and brostallicin (50 μM) for 1 h at room temperature and treated for 30 min at room temperature with a solution of MPE-$(NH_4)_2$-$Fe(SO_4)_2$ (synthesised by Pharmacia, Italy, according to the published method; Hertzberg and Dervan, 1984). After precipitation, DNA was resuspended in loading buffer and electrophoresed on an 8% polyacrylamide 7 M urea gel and autoradiographed.

Taq polymerase stop assay

Studies with the Taq stop assay were based on a previously reported method (Ponti *et al*, 1991). Prior to drug-DNA

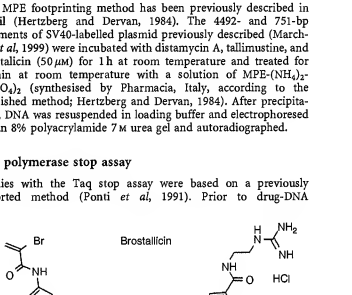

Figure 1 Chemical structures of brostallicin and tallimustine. Both molecules share the distamycin A backbone.

incubation, plasmid pBSSK-TOPO II was linearised with a *Pst*I restriction enzyme (NEB) to provide a stop for the Taq polymerase, downstream from the primer. After drug treatment, the DNA was precipitated and washed as described (Ponti *et al*, 1991). The primer was 5′-end labelled with T4 polynucleotide kinase (NEB) and [γ-^{32}P] ATP (5000 Ci mmol^{-1}, Amersham). The synthetic primer sequence and the linear PCR amplification conditions were performed as described (Marchini *et al*, 1998). Samples were then purified by extraction with an equal volume of chloroform–isoamyl alcohol (24 : 1), and then precipitated and washed following the standard protocol. Dried samples were resuspended in loading buffer and denatured at 90°C for 2 min before loading onto an 8% polyacrylamide denatured gel. After the run, the gel was dried and autoradiographed.

Clonogenic survival and MTT proliferation assays

Clonogenic survival in response to drug treatment was performed by plating 250 cells in 60 mm cell culture dishes. After 24 h, the drug was added, followed by incubation in a drug-containing medium for 2 h or 24 h and then in a drug-free medium for another 6–8 days at 37°C in a humidified atmosphere containing 5% carbon dioxide. Cells were then fixed with 25% acetic acid in ethanol and stained with Giemsa. Colonies of at least 50 cells were scored visually. Each experiment was performed a minimum of three times using triplicate cultures for each drug concentration. The logarithm of relative colony formation was plotted against the concentration of the drug. The IC_{50} was estimated by linear interpolation of the logarithmic transformed relative plating efficiencies.

For ATM$^{+/+}$/p53$^{-/-}$ and ATM$^{-/-}$/p53$^{-/-}$ mouse cells that do not form distinct colonies, the drug sensitivity was determined by the MTT assay (Mosmann, 1983). MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide) measures the mitochondrial dehydrogenase of surviving cells. Cells growing in the log phase were harvested by brief trypsinisation. A total of 1000 cells were plated (96 well plates) 24 h prior to 2 h drug treatment. Cells were then grown in a drug-free medium for another 4 days at 37°C in a humidified atmosphere containing 5% carbon dioxide. A volume of 20 μl MTT in PBS to a final concentration of 0.5 mg ml^{-1} was added, followed by incubation at 37°C for 4 h, aspiration of the medium, and addition of 200 μl DMSO. The optical density was measured by the E_{max} microplate reader E9336 (Molecular Devices, Clearwater, MN, USA) at 540 nm, setting the value of the cell lines in the medium to 1.0 (control) and the value of the no cells blank to zero. Differences in drug sensitivity of the respective cell lines were determined from at least four independent experiments and are reported as the concentration required to suppress proliferation by 50% (IC_{50}).

Statistical analysis

The mean $\pm$ s.d. values were calculated for all data sets. The two-sided paired *t*-test was used to compare the effects on drug sensitivity. $P < 0.05$ was considered to be statistically significant.

RESULTS

Brostallicin does not alkylate DNA *per se* but through the interaction with GSH/GST

Noncovalent interactions of brostallicin and tallimustine (TAM) with DNA were compared to those of distamycin A (DISTA). The data reported in Figure 2 show an autoradiograph of a classical ladder of an MPE-footprinting experiment tested on the 751-bp (panel A) and 4492-bp fragments (panel B) of the SV40 DNA plasmid. Each band corresponds to a DNA fragment differing in size by a single nucleotide. When a DNA region is protected by the

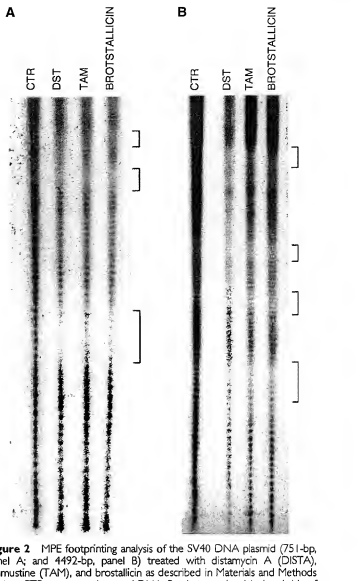

Figure 2 MPE footprinting analysis of the SV40 DNA plasmid (751-bp, panel A; and 4492-bp, panel B) treated with distamycin A (DISTA), tallimustine (TAM), and brostallicin as described in Materials and Methods section. CTR = untreated control DNA. Brackets on the right-hand side of the picture refer to AT-rich regions determined by DNA sequencing.

presence of a molecule, chemical digestion is blocked and a 'gap' is present on the autoradiograph. In the control (CTR, Figure 2), all fragments are present with broadly the same signal intensity on the gel, while in all the treated sample lanes a typical 'gap' is common in AT-rich regions. The brackets highlight these regions. The distamycin A backbone present in the brostallicin chemical structure drives the DNA interaction towards AT-rich regions in the same way as previously shown for tallimustine. In fact, brostallicin shows a noncovalent DNA interaction effect superimposable to that of tallimustine and distamycin A (internal positive control). These regions are highlighted by brackets. The differences in band intensities were due to differences in gel loading.

On the basis of the previously reported data showing that brostallicin is able to covalently interact with DNA upon *in vitro* reduction by the GSH/GST system (Geroni *et al*, 2002), we further tested this hypothesis by incubating the drug-DNA solution with and without GSH and GST in an *in vitro* system. The sequence-specific, covalent DNA interaction of brostallicin in comparison with tallimustine was analysed by the Taq polymerase stop assay. This assay is a linear amplification method employing the

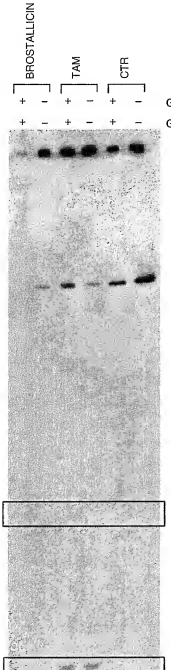

Figure 3 Autoradiography of a typical Taq Stop Assay on topoisomerase II cDNA after treatment with brostallicin and tallimustine (TAM). The experiment was performed as described in Materials and Methods section. CTR = control untreated sample. Arrows indicate the alkylated regions.

properties of DNA polymerase to investigate the sequence selectivity of the interaction between DNA-damaging agents and the DNA. As expected, tallimustine retained its high sequence specificity in alkylating DNA at the target hexamer (5′-TTTTGA), while brostallicin *per se* was completely unable to produce any alkylation in the selected DNA region (Figure 3). Brostallicin alone did not alkylate DNA, while a band was observed when GST/GSH was added to the reaction mixture. In the absence of brostallicin, GSH/GST did not induce any alteration able to block Taq polymerase. It is important to underline the fact that, although the interaction of brostallicin with DNA involves AT-rich regions, the compound binds to a sequence (AAAG) different from those previously reported for tallimustine. Studies are still in progress to better define the sequence of the alkylated regions.

Table 1 IC_{50} concentrations for clonogenic survival of MMR-proficient or -deficient cells in response to treatment with brostallicin or tallimustine

Compound	Cell line	IC_{50} (nM)	Ratio[a]	P[b]
Brostallicin	HCT116+ch3	13.1 ± 3.2		
	HCT116par	14.9 ± 2.8	1.14	0.36
	HEC59+ch2	28.7 ± 4.5		
	HEC59par	35.0 ± 9.9	1.22	0.41
Tallimustine	HCT116+ch3	44.3 ± 3.9		
	HCT116par	133.5 ± 6.4	3.06	<0.01
	HEC59+ch2	13.1 ± 2.6		
	HEC59par	24.0 ± 4.6	1.83	0.03

Mean ± s.d. of at least three independent data sets. [a]Ratio of IC_{50} values of MMR-deficient cells and -proficient cells. [b]Two-sided paired *t*-test.

Loss of MLH1 or MSH2 does not alter sensitivity to brostallicin

The question was addressed as to whether loss of either MLH1 or MSH2 affects the sensitivity to brostallicin using the clonogenic assay. The data presented in Table 1 show that MLH1-deficient HCT116 cells are nearly as sensitive as MLH1-proficient HCT116 + ch3 cells to this drug ($P = 0.36$). This indicates that MLH1 is not involved in brostallicin-mediated cytotoxicity. Furthermore, MSH2-deficient HEC59 cells are as sensitive to brostallicin as MSH2-proficient HEC59 + ch2 cells ($P = 0.41$), indicating that brostallicin-mediated cytotoxicity does not require functional MSH2. Brostallicin cytotoxicity has been compared to tallimustine. The results show that MLH1-deficient and MSH2-deficient cells are three-fold ($P < 0.01$) and 1.8-fold ($P = 0.03$), respectively, less sensitive to tallimustine than their respective proficient counterparts.

Sensitivity to brostallicin, but not to tallimustine, is retained after loss of PMS2

Although less frequently mutated than MLH1 or MSH2 in human cancers, PMS2 may nevertheless be relevant in this respect since it forms a heterodimer with MLH1 and the lack of one or the other partner affects MMR activity. Based on the model that cytotoxicity of tallimustine, but not the α-bromoacrylic derivatives, is dependent on functional MMR, it is anticipated that loss of PMS2 negatively affects sensitivity to tallimustine, but not to brostallicin. The effect of loss of PMS2 on drug sensitivity was investigated in p53-deficient cells derived from knockout mice. Table 2 shows that the clonogenic survival after treatment with brostallicin in PMS2-deficient cells was not different from that in PMS2-proficient cells ($P = 0.79$). In contrast, PMS2-deficient cells were 1.6-fold less sensitive to tallimustine than PMS2-proficient cells ($P = 0.02$).

Thus, PMS2-deficient p53-null mouse fibroblasts retain sensitivity to brostallicin. The 1.6-fold resistance to tallimustine in PMS2-deficient cells indicates a role for PMS2 in sensitivity to this compound.

Loss of ATM or DNA-PK does not affect sensitivity to brostallicin

It has previously been proposed that the cytotoxic effect of the α-bromoacrylic derivative PNU-151807 interferes with the cell cycle

Table 2 IC_{50} concentrations for clonogenic survival of PMS2-proficient or -deficient mouse cells in a p53-deficient genetic setting in response to drug treatment

Drug	$PMS2^{+/+}/p53^{-/-}$	$PMS2^{-/-}/p53^{-/-}$	Ratio[a]	P[b]
Brostallicin (nM)	27.3 ± 2.8	26.3 ± 6.7	0.96	0.79
Tallimustine (nM)	1.39 ± 19	223 ± 42	1.60	0.02

Mean ± s.d. of at least three independent data sets. [a]Ratio of IC_{50} values of MMR-deficient cells and -proficient cells. [b]Two-sided paired *t*-test.

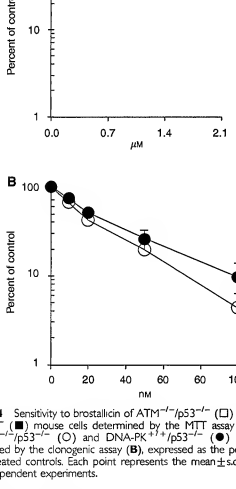

Figure 4 Sensitivity to brostallicin of $ATM^{-/-}/p53^{-/-}$ (□) and $ATM^{+/+}/p53^{-/-}$ (■) mouse cells determined by the MTT assay (**A**) and of DNA-$PK^{-/-}/p53^{-/-}$ (○) and DNA-$PK^{+/+}/p53^{-/-}$ (●) mouse cells determined by the clonogenic assay (**B**), expressed as the percentage of the untreated controls. Each point represents the mean ± s.d. of at least four independent experiments.

checkpoint control (Marchini *et al*, 1999). Although yet unknown, a possible pathway may include ATM or DNA-PK, members of the PI3-like kinase family, which are important kinases for connecting DNA damage monitoring and cellular responses such as cell cycle checkpoint activation and apoptosis. The question was addressed as to whether the sensitivity to brostallicin is affected by loss of ATM or DNA-PK in a p53-deficient genetic background. We used embryonic fibroblasts from knockout mice. The data presented in Figure 4 show that ATM-deficient cells (0.8 ± 0.3 μM) were as sensitive to brostallicin as ATM-proficient cells (0.9 ± 0.2 μM) in a p53-deficient genetic setting ($P = 0.60$). Likewise, DNA-PK-deficient cells (17.5 ± 0.7 nM) were as sensitive to brostallicin as DNA-PK-proficient cells (21.0 ± 1.4 nM) in a p53-null background ($P = 0.13$).

Thus, neither ATM nor DNA-PK seems to be involved in the sensitivity to brostallicin in p53-deficient mouse cells.

DISCUSSION

The present study demonstrates that brostallicin, a novel α-bromoacrylic, second-generation DNA MGB structurally related to distamycin A, maintains its cytotoxic effect in cells deficient for the MMR proteins MLH1, MSH2, or PMS2. The data permit drawing several conclusions. First, brostallicin, the lead compound of a novel class of MGBs in clinical trials, exerts its cytotoxic effect regardless of the MMR status, suggesting that further clinical testing of brostallicin in tumours deficient in MMR is to be recommended. Second, brostallicin-induced cytotoxicity can occur in the absence of functional ATM or DNA-PK in p53-deficient cells, indicating that brostallicin-induced cytotoxicity in this setting is independent of PI3-like kinase family status. Third, brostallicin is the first MGB unable to *per se* covalently interact with DNA. It requires the GSH/GST system to alkylate DNA with a sequence specificity different from that reported for previously tested alkylating molecules.

MMR plays an important role in the correction of spontaneously occurring errors during DNA processing that have escaped the DNA polymerase proof-reading activity, thereby preserving the integrity of the genome by preventing the occurrence of gene mutations and tumorigenesis (Modrich, 1991). Spontaneous tumours arising from MMR deficiency include the hereditary nonpolyposis colon cancer as well as some sporadic carcinomas such as mammary, ovarian, or endometrial cancers (Peltomaki, 2001). MMR monitors specific types of DNA damage introduced by DNA-damaging agents, and subsequently triggers an apoptotic response (Fink *et al*, 1998). Loss of MMR hence results in resistance to a variety of widely used anticancer drugs, including the topoisomerase I poisons camptothecin and topotecan, the topoisomerase II poisons doxorubicin, epirubicin, mitoxantrone and etoposide, and some platinum compounds such as cisplatin and carboplatin, as well as some alkylating agents including MNNG and busulphan (Branch *et al*, 1995; Drummond *et al*, 1996; Fink *et al*, 1996, 1998; Fedier *et al*, 2001).

Interestingly, the MMR status also affects the activity of several MGBs such as CC-1065 analogues and the distamycin-derivative tallimustine, but not that of the α-bromoacryoyl derivative of distamycin A (PNU-151807) (Colella *et al*, 1999). The present study expands on this previous finding by demonstrating that brostallicin, a novel second-generation DNA MGB structurally related to PNU-151807, exerts its cytotoxic effect regardless of the MMR status. Brostallicin as well as the class of the taxanes (Fedier *et al*, 2001) and photodynamic therapy (Schwarz *et al*, 2002) may thus represent valuable options for the treatment of tumours disabled in MMR.

The α-bromoacryoyl moiety has been proposed to be important since it reacts with GSH, and reactive drug-GSH intermediates may then modify the DNA by mechanisms not yet fully understood (Geroni *et al*, 2002; Cozzi, 2003). DNA interaction data reported in the present study suggest that the distamycin A backbone drives the brostallicin molecule towards the AT regions present in the minor groove of the DNA. In addition, brostallicin binds covalently to DNA through interaction with the GSH/GST system. Brostallicin covalently binds to DNA with a completely different sequence specificity than tallimustine. One hypothesis for the different behaviour of brostallicin against MMR status is that this covalent interaction is not substrate for MMR, whereas the alkylated DNA by tallimustine is recognised by MMR. It should be noted that no direct interaction between MMR and the GSH/GST system is known, and that the GSH/GST status of the cell lines

under study does not matter for the experiments because the cell lines are quasi-isogenic, that is, they differ only in their MMR status and the extra chromosomes.

Moreover, as reported for PNU-151807, the bromoacryloyl moiety seems to be relevant for cell cycle checkpoint control (Marchini *et al*, 1999). The identity of mediators for signalling between DNA damage and downstream effectors is not clear. One possibility is that the DNA damage is recognised by one or several members of BASC (BRCA1-associated genome surveillance complex), a multiprotein complex including BRCA1, ATM, MMR proteins, and other proteins implicated in DNA repair (Wang *et al*, 2000). Our data, however, show that deficiency in ATM or DNA-PK did not affect brostallicin sensitivity in p53-deficient cells, arguing against a role of these kinases in these cells. Since these kinases are activated upon DNA double-strand breaks introduced by radiation or radiomimetic drugs (Jackson, 1997; Smith *et al*, 1999), α-bromoacryoyl derivatives seem unlikely to produce this type of lesion. Although the cytotoxic effect of tallimustine and PNU-151807 has been shown not to be dependent on the p53 status (Marchini *et al*, 1999), the data for these kinases obtained in p53-deficient cells may not be conclusive for p53-proficient cells. There is an apparent higher sensitivity to brostallicin of the DNA-PK data set compared to the ATM data set, but this is likely due to the use of two assays that differ in their sensitivities.

Mutations in the p53 tumour suppressor gene are found in a large fraction of human cancers (Hollstein *et al*, 1991) and this may be the genetic basis underlying failure to respond to chemotherapy (Ferreira *et al*, 1999). PNU-151807 has recently been reported to retain sensitivity against cells disabled in p53 function (Marchini *et al*, 1999), indicating that PNU-151807-mediated cytotoxicity does not require functional p53. We have recently shown that additional loss of PMS2 in p53-deficient cells increases cytotoxicity to a variety of anticancer agents (Fedier *et al*, 2002). This hypersensitising effect, however, was not observed in response to treatment with brostallicin. For tallimustine, even an opposite effect was observed in PMS2-deficient cells, suggesting that tallimustine-induced DNA damage is a substrate for MMR in p53-deficient cells. Consistent with this, tallimustine-induced DNA damage has already been shown to be a substrate for MMR in p53-proficient cells (Colella *et al*, 1999).

We also observed that tallimustine is less toxic than brostallicin in p53-deficient cells and that this effect is much greater than the difference in sensitivity to tallimustine between MMR-deficient and -proficient cells. This marked effect was not observed in p53-proficient cells. As the status of p53 has been reported not to markedly affect the sensitivity of human tumour cells to either tallimustine or PNU-151807 (Marchini *et al*, 1998), this effect in p53-deficient cells may be ascribed to the mouse origin and/or to the fibroblast cell type.

In summary, the present study demonstrates that brostallicin-mediated cytotoxicity does not depend on the MMR status of tumour cells, and that, at least in p53-deficient mouse cells, functional ATM or DNA-PK is not required. Brostallicin potentially offers the advantage of having efficacy on MMR-defective tumours that are refractory to several anticancer agents. Since the responsiveness to cisplatin treatment is affected by both MMR status and GSH/GST level/expression, brostallicin is a good candidate for clinical protocols.

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